

Pseudoexfoliation deposits adhering on the anterior human lens capsule consists of autofluorescent proteinaceous extracellular matrix and impacts lens homeostasis

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Purpose: Pseudoexfoliation syndrome (PEX) is a systemic, age-related disease most likely caused by mutations in the LOXL1 gene and is frequently diagnosed by early-onset cataracts with distinctive deposits on the lens capsule. The aim of this study was to examine the lens remnants recovered during cataract surgery to elucidate the mechanisms underlying these changes.

Methods: Surgical debris of cataract patients with PEX (PEX) were compared with those of cataract patients with diabetes mellitus (DM), another age-related disease, and cataract patients without both diagnoses (control). The anterior lens capsule piece was investigated by nonlinear two photon emitted autofluorescence (TPEF) and fluorescence lifetime imaging (FLIM) measurements. Advanced glycation end products (AGEs) and crystallin gene expression were measured in the phacoemulsification solution.

Results: In the PEX-group, the AGEs content was significantly higher than in DM and control group and correlated positively with age. TPEF revealed only a loose attachment of flakes on the PEX lens capsules. Using FLIM the PEX material could be characterized by a specific lifetime differing significantly from those of the lens capsule and epithelial cells. PEX and DM group showed significantly higher crystallin β B2 gene expression compared to control. Although the total expression of crystallin α A and α B was the same in PEX and control group, the ratio of α A and α B was significantly lower in the PEX group.

Conclusions: Cataract in PEX patients results from altered crystallin expression evoked by the overall connective tissue changes (α B upregulation) and the deposition of the flakes on the capsule (β B2 upregulation).

INTRODUCTION

Cataract affects almost every human being without exception in old age, besides there are many infantile cataracts especially in developing countries. The development of cataracts is not yet finally understood. It can be favored by factors like diabetes mellitus.¹ The human lens can be roughly divided into nucleus and cortex. In the area of the anterior surface the single layered lens epithelium appears under the capsule, further towards the lens equator cell division occurs in the germinative zone in the area of the equator the cells transform into fiber cells. These lose their nuclei in the further course and are ligated by subsequent fiber cells in the direction of the nucleus. During this lifelong ongoing process, crystallins accumulate, which are essential for the refractive power and transparency of the lens. α , β and γ crystallin are the most frequently occurring classes in the lens. The α crystallin prevail, which have high structural similarities with small heat shock proteins and were therefore suggested to act as chaperones.² Consequently, ageing and cataract formation were assumed to be the result of decreasing chaperone activity.² However, other studies assume that misfolding due to polymorphisms and mutations affects all crystallin classes and favors cataracts.^{3,4} Clinically, cataracts are often diagnosed alongside another age-related disease, namely Pseudoexfoliation syndrome (PEX).

PEX is a systematic disease diagnosed mainly by visible changes in the eye. Fine fibrils are seen in the eye on the anterior surface of the lens and the artificial intraocular lens in pseudophakic eyes (**Figure 1 A, B**), which can often be incidental findings and asymptomatic. The deposits are associated with an increased risk of glaucoma and zonular weakness. The latter could increase the risk of zonulolysis, when cataract surgery is performed an lead to subluxation of the intraocular lens (**Figure 1 C, D**) requiring repeated surgery.⁵ Various causes for PEX have been discussed, of which mutations in the LOXL1 gene are the most likely.^{6,7} PEX fibrils can be detected posthumously throughout the body of affected patients and are linked to pulmonal, urogenital and cardiovascular diseases.^{8,9}

There is some theoretical and experimental evidence for a link between PEX and advanced glycation end products (AGEs). The non-enzymatic glycation of proteins leads to an irreversibly altered product via stabilization by means of Amadori rearrangement. As the physiological stabilization of the extracellular matrix, e.g. collagen and elastin, takes place via the same chemical process, changes in the continuous remodeling process also result in modified proteins, which are classified as AGEs. For this reason, N(6)-Carboxymethyllysine (CML) is often used as a standard marker in the analysis of AGEs. By immunohistochemical analysis, AGE CML was detectable in human lens capsules¹⁰. Since LOXL1 encodes a member of the lysyl oxidase gene family, which is essential for the physiological stabilization of the extracellular matrix, a correlation of increasing AGEs due to LOXL1 mutations is conceivable. Many AGEs show autofluorescence, which is increasingly being used to diagnose them. In a Japanese population, skin autofluorescence measurements showed a significantly increased accumulation of

AGEs in the finger skin of patients with PEX glaucoma compared to healthy patients and patients with open angle glaucoma,^{11,12} which further supports the hypothesis. Similar processes can also be induced by oxidative stress, which has been shown to be a contributing factor in PEX.⁶

Elevated AGEs are associated with Diabetes mellitus due to the increased blood glucose level. The best-known parameter is HbA1c,¹³ which has become established worldwide for monitoring the disease. Neither elevated blood glucose nor HbA1c levels were diagnosed in patients with PEX.¹⁴ Furthermore, studies and a meta-analysis indicate that there is no overall correlation between Diabetes mellitus and PEX (Wood et al., 2011; Yu et al., 2024).^{15,16} In older population groups, however, an inverse relationship between Diabetes mellitus and PEX cannot be ruled out.¹⁶

At the molecular level, PEX fibrils represents a complex, elastotic extracellular matrix aggregate. Proteomic, ultrastructural, and transcriptomic studies have shown that it contains fibrillin 1 and other microfibril components, latent TGF β binding proteins, fibronectin, vitronectin, complement factors, apolipoproteins, LOXL1, and various basement membrane and elastic fiber-associated glycoproteins. These changes are accompanied by dysregulation of extracellular matrix organization, TGF β signaling, and oxidative stress pathways in the lens capsular epithelium.^{17,18} Oxidative damage, non-enzymatic glycation, and crosslinking of matrix proteins in PEX eyes contribute to basement membrane remodeling and may alter the biomechanical and optical properties of the anterior lens capsule. Together, these findings support one current view of PEX as a basement membrane- and elastic fiber-associated fibroproteinopathy rather than a purely localized degenerative deposit.^{19,20}

Despite extensive histopathological and biochemical characterization of PEX material on the lens capsule, current imaging approaches remain largely morphological. Slit lamp biomicroscopy and digital photography describe the macroscopic distribution and pattern of PEX deposits (**Figure 1**) but provide no direct information on their biochemical microenvironment. Anterior segment optical coherence tomography and ultra-high resolution Optical Coherence Tomography (OCT) have improved structural visualization of the capsule-zonule complex and PEX related alterations in anterior segment geometry, yet they lack intrinsic molecular contrast.^{19,21} Confocal and intraoperative endoscopic imaging offer higher spatial resolution of granular, dandruff like deposits on the capsule and zonules, but their contrast is primarily based on reflectivity and scattering.²²⁻²⁴ None of these techniques can distinguish, in a label free manner, between pseudoexfoliative material and the underlying lens capsule on the basis of endogenous molecular signatures.

Nonlinear two photon emitted autofluorescence (TPEF) measurement using multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) provides powerful, label free approaches to probe the biochemical and structural microenvironment of endogenous fluorophores in tissue. Unlike intensity based TPEF imaging, FLIM measures the fluorescence decay kinetics, which are sensitive to

local factors such as protein conformation, crosslinking, binding interactions, oxidation, and the presence of advanced glycation end products.

To gain further insights into the pathology of PEX, the aim of the study was to examine the lens remnants that regularly occur during cataract surgery using high-resolution, dye-free fluorescence microscopy (TPEF and FLIM) and molecular biological techniques. For this purpose, cataract patients with PEX were compared with cataract patients with diabetes mellitus and those without these diagnoses.

METHODS

This study was approved by the Rostock University Medicine Ethics Review Board and adheres to the tenets of the Declaration of Helsinki. Regularly occurring tissue from 29 patients who underwent medically necessary cataract surgery according to the standardised methodology at the Department of Ophthalmology, University Medical Center Rostock, Germany were included. Both the anterior lens capsule piece removed by standard capsulorrhexis of 5-5.5 mm and the phacoemulsification solution containing lens nucleus and cortex fragments were used. The anterior lens capsule piece was placed in corneal culture medium. (Merck K2 medium, F9017, supplemented with 2.5% fetal calf serum), transferred to a microscope slide and examined by TPEF and/or FLIM within 24 h after surgery. The phacoemulsification solution was temporarily stored at 4 °C, mixed thoroughly, and then distributed evenly between three tubes and centrifuged at 4000 rpm for 10 min. Two pellets were solved in 500 µl RLT buffer with mercaptoethanol and used for RNA isolation and one pellet was solved in 500 µl RIPA lysis buffer for protein and AGEs analysis.

Clinical examinations

All patients underwent complete ophthalmological examination before surgery, including slit-lamp biomicroscopy, Scheimpflug tomography (Pentacam; Oculus, Wetzlar, Germany) and OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany). In addition, the patient's medical history was taken to obtain reliable information about the presence of diabetes mellitus. Best-corrected visual acuity (BCVA) was determined with reference to the logarithm of the minimum angle of resolution (LogMAR) and central retinal thickness was calculated from OCT by spectral-domain technology. Classification into the PEX group was based on the pseudoexfoliation deposits on the anterior surface of the lens (**Figure 1**).

Protein and AGEs measurements

Samples were diluted 1:20 in phosphate buffered saline (PBS). Protein content was determined by the Bradford method. The proportion of autofluorescent AGEs in the solution was measured in a black microtiter plate in a microplate fluorimeter (Fluoroskan Ascent, Thermo Fischer Scientific) at an

excitation wavelength of 355 nm at 460 nm emission against Advanced Glycation Endproduct-BSA 10mg/ml (Cat.121800, Merck, Darmstadt, Germany) as a standard in six replicates.

Gene expression analyses

Pellets from phacoemulsification solution were homogenized in QIAshredder Mini Spin Columns (Qiagen, Hilden, Germany) by centrifugation (2 minutes, 12,000 rpm). Thereafter RNA was isolated and purified with the RNeasy Mini Kit (Qiagen). The cDNA was synthesized using the Maxima First Strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, Darmstadt, Germany). RNA solutions containing the probes of the cDNA synthesis kit were placed in a thermocycler (Labcycler; SensoQuest, Göttingen, Germany) programmed at 25°C for 10 minutes, followed by 50°C for 15 minutes and by 85°C for five minutes. For real-time PCR, cDNA solutions containing TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and TaqMan Gene Expression Assays (CRYGD Hs00359815_m1; CRYGC Hs00253214_m1; CRYBB1 Hs00157113_m1; CRYBB2 Hs00166761_m1; CRYBA1 Hs00193230_m1; CRYBA4 Hs00608089_m1; CRYAB Hs00157107_m1; CRYAA Hs00166138_m1; ACTB Hs01060665_g1) of primer and gene probe were amplified and detected using a 7900HT Fast Real-Time System (Life Technologies, Darmstadt, Germany). The PCR system was programmed at 50°C for two minutes, followed by 95°C for 10 minutes and by 100 repeats of the steps 95°C for 15 seconds and 60°C for one minute. Selection of an endogenous control for normalization was done in line with the TaqMan Gene Expression (Applied Biosystems) recommendations. Actin served as a housekeeping gene in this study. Gene expression values were calculated with the SDS RQ Manager 1.2 software (Life Technologies).

TPEF measurments

Samples were investigated using an upright dual-line FVMPE RS multiphoton microscope (Evident, Hamburg, Germany) equipped with a femtosecond laser system (InSight DS Dual-OL; Spectra-Physics, Santa Clara, CA, USA). A galvanometer scanner with 512 × 512 pixels was used with scan times of two to eight seconds per pixel. Images sized 512 × 512 to 2048 × 2048 were acquired with an XLPLN25xWMP (NA 1.05) objective at zoom 1.0 to 4.0 and cooled gallium arsenide phosphide photo-multiplier tubes. The 3D images were obtained by depth serial recordings with a step size of 0.5 to 2.0 µm. TPEF was detected with a 740 nm excitation wavelength (tunable laser line) and a BA460-500 emission filter. Whole sample maps were generated by multi-area time-lapse imaging. Final images were generated by Imaris software (Oxford Instruments/Bitplane, Zurich, Switzerland).

FLIM measurements and data analyses

Time-resolved fluorescence imaging was performed using a MicroTime 200 confocal FLIM system (PicoQuant, Berlin, Germany) based on an inverted microscope (IX73, Evident) equipped with a 60×/1.2 NA water-immersion objective (UPlanSApo, Evident). Excitation was provided by a ps-pulsed diode

laser operating at 375 nm with a pulse width <70 ps and a repetition rate of 20 MHz. The excitation path included a dichroic mirror, and emission was collected through a 50 μ m pinhole and a 405 nm long-pass filter and detected by a hybrid PMT detector (PMA Hybrid 40, PicoQuant). Photon arrival times were recorded in time-correlated single-photon counting (TCSPC) mode using a HydraHarp 400 module (PicoQuant). The excitation power was adjusted to maintain average count rates $\leq 10^5$ photons/s to avoid pile-up artifacts. Image acquisition was carried out with a FLIMbee galvo scanner, and data were processed using SymPhoTime 64 software (v2.7, PicoQuant).

Time-resolved fluorescence data were acquired and analyzed using SymPhoTime 64 (v2.7, PicoQuant, Berlin, Germany), which integrates instrument control and classical lifetime fitting based on iterative reconvolution with the instrument response function. Post-processing was performed in NovaFLIM (PicoQuant), a dedicated analysis platform supporting exponential fitting, phasor analysis, and pattern-matching approaches. In the phasor framework, the time-resolved decay at each pixel is transformed into a point with coordinates (g,s) in a two-dimensional sine-cosine (phasor) space, providing a global, fit-free representation of the lifetime distribution within the image. Single-exponential decays lie on the universal semicircle, with short lifetimes toward the right and long lifetimes toward the left, whereas mixtures fall inside the semicircle as linear combinations of their components. NovaFLIM exploits the reciprocity principle of phasor analysis: user-defined regions in the phasor plot ("patterns") are mapped back onto the FLIM image, enabling segmentation of pixels with similar decay characteristics. For each pattern, intensity-weighted mean lifetimes were extracted to quantify the lifetimes of different tissue areas.

Statistical analyses

Results are presented as means $\pm$ SEM. Statistical analyses were performed using the Prism 10 analysis program (GraphPad, San Diego, CA, USA). Differences were examined using one-way ANOVA with Bonferroni's multiple comparisons test or Student's *t*-test and *P* values <0.05 were considered to be statistically significant. Correlations were evaluated using the Pearson correlation coefficient.

RESULTS

AGEs levels in the eye lens of patients with and without PEX or diabetes

In total, material from 29 patients who had undergone cataract surgery were analyzed in the study. The patients were divided into three groups. 10 patients (mean age 72 ± 2 years) were included who had been diagnosed with PEX using a slit-lamp biomicroscopy (PEX group). 8 patients (mean age 78 ± 3 years) had a confirmed history of diabetes mellitus (diabetes group). The other 11 patients (mean age 75 ± 3 years) had no diagnosis of PEX or diabetes, nor were any other eye pathologies detected during the examination (control group). Compared to patients without diabetes mellitus, AGE levels in

patients with diabetes correlated positively with age (**Figure 2A**). While very high AGE levels were measured in some patients with diabetes mellitus, the mean value in the diabetes group did not differ significantly from the control group (**Figure 2B**). In patients with PEX, the AGE content was not only significantly higher than in the other two groups (**Figure 2B**), but also correlated significantly positively with age (Pearson's r : 0.79) (**Figure 2A**). Neither best-corrected visual acuity nor mean central retinal thickness differed between the groups (**Figure 2C and D**).

Crystallin gene expression in the eye lens of patients with and without PEX or diabetes

The PEX group showed significantly lower expression of the crystallin α A gene (CRYAA) compared to the diabetes and control group (**Figure 3A**). In contrast, the crystallin α B gene (CRYAB) expression was significant higher in PEX group compared to the control group. Higher expression was also observed in the diabetes group than in the control group (**Figure 3B**). Expression of the crystallin β B1 gene (CRYBB1) was significantly higher in the diabetes group compared to control and PEX group (**Figure 3C**). Both, the PEX and the diabetes group showed significantly higher crystallin β B2 gene (CRYBB2) expression compared to the control group (**Figure 3D**). Crystallin γ C gene (CRYGC) expression was significantly higher in the PEX group compared to control and diabetes group (**Figure 3E**). Gene expression of crystallin β A1 gene (CRYBA1), β A4 gene (CRYBA4) and γ D gene (CRYGD) did not differ between the groups (data not shown). Although the total expression of CRYAA and CRYAB was the same in PEX and control group (**Figure 4A**), the ratio of CRYAA to CRYAB was significantly lower in the PEX group (**Figure 4B**). On an individual basis, it was found that patients with high total expression of the crystalline proteins exhibited the largest imbalance in comparison to the control group. CRYAA expression correlated significantly negatively with the sum of CRYAA and CRYAB expression in the PEX group (Pearson's r : -0.73) (**Figure 4C**).

Characterisation of the anterior lens capsule by TPEF

Both the lens capsule and the epithelial cells exhibited significant autofluorescence, which could be used for their visualization using multiphoton microscopy (**Figure 5 and 6**). No significant difference between the groups could be detected in the structure of the epithelial cells, which is represented on the inside of the lens capsule by the characteristic NAD(P)H autofluorescence of the mitochondria (**Figure 5 A-C**). It should be noted that different confluence of the cells results from the detachment of the epithelium from the fiber cells during surgery. While the outer surface of the lens in the control group appeared as a uniform, smooth surface (data not shown), deposits of varying density were visible in the PEX group (**Figure 5 D-E and Figure 6**). In some cases, the material in the samples was also found in solution (**Figure 6A**). Detailed images (**Figure 6B-C**) showed a flake-like shape with strong autofluorescence.

Characterisation of the anterior lens capsule by FLIM

Comparable to TPEF, lens epithelial cells at the inner side of the anterior lens capsule could be visualized using FLIM (**Figure 7**). Phasor analysis (**Figure 7D**) identified two main components in a sample of the control group: Pattern 1 (blue) (**Figure 7C**), corresponding predominantly to mitochondria of epithelial cells with a mean lifetime of $\tau_1 \approx 3.3$ ns, and Pattern 2 (red) (**Figure 7B**), localized mainly to the capsule/basement membrane with a longer mean lifetime of $\tau_2 \approx 3.9$ ns. The reproducibility of this lifetime pattern across eyes and fields of view could be confirmed in further control samples (**Figure 7E-F**). The PEX fibrils were detectable exclusively on the outside of the anterior lens capsule as discrete crystal like or granular structures, either as isolated particles or clustered aggregates distributed heterogeneously across the capsule, rather than continuous fibrillar sheets (**Figure 8**). Lifetime analyses (**Figure 8A**) reveals two main lifetime domains within this material, with blue regions in the pseudocolor lifetime map indicating shorter lifetimes and red regions indicating longer lifetimes, suggesting biochemical micro heterogeneity within the aggregates. Two component pattern matching and phasor FLIM analysis (**Figure 8D**) enabled separation of the PEX material from the native lens capsule matrix. Pattern 1 (blue, $\tau \approx 3.8$ ns) (**Figure 8C**) co localized with the granular PEX deposits on the capsule surface, whereas pattern 2 (red, $\tau \approx 4.2$ ns) (**Figure 8B**) mapped to the underlying capsule basement membrane. Again, the matrix associated lifetime was longer than those measured in co-imaged lens epithelial cells (mitochondria: $\tau \approx 3.3$ ns; cytoplasm: $\tau = 3.9$ ns, data not shown). This was confirmed in further samples from the PEX group. In line with the TPEF measurements, PEX deposits on the anterior lens capsule are highly heterogeneous in their spatial distribution and organization (**Figure 8E-F**). PEX material appears either as isolated, discrete particles or as clustered aggregates confined to specific regions of the capsule surface, rather than as a homogeneous, continuous layer. Altogether PEX deposits showed a lifetime that tended to be shorter than that of the cytoplasm of the lens epithelial cells but differed significantly from that of the mitochondria of the lens epithelial cells and that of the lens capsule (**Figure 9**).

Cross sectional FLIM imaging became possible at the cut edge of the anterior lens capsule of a 65 years old patient with PEX. This image setting allowed visualisation of PEX material and lens epithelial cells on both sides of the capsule surface in one view and thus, a direct comparison of the lifetime of all three components. At the capsule margin (**Figure 10A, D**), pattern matching separates a superficial PEX layer (Pattern 1, blue, $\tau \approx 4$ ns) from the cell on the middle (Pattern 2, red, $\tau \approx 4.7$ ns) and the underlying capsule basement membrane (Pattern 3, green, $\tau \approx 4.9$ ns). In another field from the same sample (**Figure 10B, D**), pattern matching separated capsule matrix ($\tau \approx 4.6$ ns, Pattern 3, green) from PEX material ($\tau \approx 4$ ns, Pattern 1, blue) and lens epithelial cell ($\tau \approx 4.4$ ns, Pattern 2, red). In the peripheral zoom (**Figure 10C, D**), epithelial cells ($\tau \approx 4.2$ ns) and capsule matrix ($\tau \approx 4.8$ ns) are separated by pattern matching. The clearly resolved subcellular structures correspond mainly to cytoplasm, as their lifetime

(≈ 4.2 ns) is close to the cytoplasmic lifetime in **Figure 7B** (≈ 3.9 ns), rather than to the mitochondrial lifetime.

DISCUSSION

While the number of AGEs in the eye lens in patients with diabetes mellitus correlated positively with age, this correlation could not be demonstrated in cataract patients without diabetes. This is in line with the published literature.²⁵ However, the AGE content in the eye lens showed high variability in our patient group randomly selected for all diabetes mellitus pathologies. Further analyses would require a larger patient cohort and selection according to diabetes type, age of onset, and therapy. However, this study focused on the comparison with the group of cataract patients with PEX. We were able to show that the AGE content in the group of patients with PEX was significantly higher than in the group of patients with diabetes mellitus and in the control group. In addition, the amount of AGEs detected increased significantly with age. This is an interesting finding that could support the hypothesis that the disease is associated with increased oxidative stress and an accumulation of oxidized proteins. However, it remains to be seen whether these are mainly AGEs. In PEX patients with exfoliative glaucoma, in the epithelial cells of the lens capsule the AGE carboxymethyllysine could be detected, but not in normal cataractic capsules.¹⁰ The photometric analysis used in this study, as in many other studies on the detection of AGEs¹², generally detects proteins with strong autofluorescence.

The imaging modalities TPEF and FLIM are based on the endogenous autofluorescence of proteins. By both microscopy techniques, we were able to successfully detect PEX deposits as flaky material adhering to the outside of the anterior lens capsule (**Figure 11**). Since floating material was also visible, it is very likely that during surgery, some of the PEX material ends up in the phacoemulsification solution and is ultimately detectable as fluorescent proteins in the AGEs measurement. Since PEX is a progressive disease, the deposits accumulate in the body throughout life. This explains why we were able to detect increasing amounts with advancing age. In addition, the TPEF and FLIM images showed that the PEX material is located only on the outside of the anterior lens capsule and is not connected to either the epithelial cells or the lens capsule matrix. Detailed analyses of the FLIM measurements showed that the PEX material has specific properties that differ from those of the surrounding tissue. Typical cellular NAD(P)H FLIM studies report free and protein-bound components of approximately 0.3–0.5 ns and 1.5–3.0 ns, respectively with mitochondria-rich regions (e.g. mitochondria of the lens epithelial cells) shifted toward the long component (3.5 ns), whereas structural proteins, cross-linked collagen, advanced glycation end products, and lens-specific fluorophores can exhibit multi-nanosecond lifetimes in tissue.^{26,27} Accordingly, the elevated mean lifetimes observed in both, the cytoplasm of epithelial cells and the lens capsule (4.1 and 4.4 ns, respectively) are consistent with

complex autofluorescence in cells and tissue dominated by multiple proteinaceous and matrix-associated fluorophores.^{28,29}

In PEX-affected samples, two-component phasor FLIM with pattern matching revealed that PEX deposits show moderately prolonged lifetimes (3.7 ns), thus remaining within the upper range described for cross linked or glycation modified proteins in biological matrices. However, because this lifetime differs significantly from both, the lens capsule and the mitochondria-rich epithelial lens cells it supports the interpretation that PEX deposits represent an abnormal, highly proteinaceous extracellular matrix accumulation with an altered molecular composition and microenvironment adhering to the anterior surface of the lens. It can be assumed that this occurs due to physicochemical properties at increasing concentrations of PEX flakes in the aqueous humour (**Figure 11**). The apparent comparability of the lifetime of PEX material with that of cytoplasm suggests that the material could consist of cell remnants. This is in line with the concept of a multi tissue origin of PEX material driven by dysregulated extracellular matrix metabolism due to LOXL1 mutations.^{30,31}

The elevated blood glucose level in patients with diabetes mellitus leads to an increase in the glucose content in the aqueous humour. Over time, this will affect the lens of the eye and exert an increased growth stimulus on the epithelial cells³². We were able to demonstrate a significant effect on the gene expression of the crystallin family in the eye lens. The crystallin β B family is highly expressed but less specific to the eye lens and are produced throughout the body. There are indications that they may act as stress proteins. Increased expression could therefore be a clear indication of altered metabolism in the diabetic eye lens. Crystallin α B acts primarily as a chaperone³³, preventing the aggregation of beta and gamma crystallin's and thus counteracting cataract formation. The increased content, in line with the increased crystallin β B gene expression, indicates oxidative stress caused by elevated glucose, which is to be compensated for by elevated Crystallin α B.

Compared to the control group of similar age with cataracts, patients with PEX also showed a different gene expression pattern of the crystallins in the eye lens.³⁴ The significant increase in the gene expression of the stress protein crystallin β B2 and the age-unspecific fivefold increase in the expression of γ C, a crystallin that is necessary for the highly ordered, compact structures that ensure the strength and transparency of the lens, suggest that the function of the eye lens is negatively affected by the deposits and that an adaptive response occurs⁴. Ultimately, this is not counteracted by an increased chaperone concentration, as although more crystallin α B is formed, less crystallin α A is produced. For crystallin β B2 a significant increase could be shown in protein analyses of aqueous humor from PEX patients,³⁵ which is in line with the higher gene expression found in this study in the PEX group.

Although α A and α B are proteins belonging to the same subgroup of the crystallin family, they have specific functions as molecular chaperones. In the eye lens, α A and α B occur in a ratio of about 3:1 and

predominantly form hetero-oligomeric complexes (**Figure 4D**).^{36–40} Within this complex, optimal physical properties are achieved to maintain the solubility of the lens proteins throughout life. Outside the eye lens, however, α B fulfils its chaperone function as a homo-oligomer or in cooperation with other heat shock proteins. This difference can also be explained by the fact that in the eye lens proteins cannot be degraded and thus, are not subject to regular turnover.

An interesting finding emerged when the ratio of α A to α B was examined on an individual patient basis. The physiologically described dominance of α A is no longer present in all investigated patients with cataract. However, whilst the ratio between α A and α B was still almost the same in patients with cataract alone, a shift towards a higher α B concentration was observed in patients with cataract and PEX. Thus, it might be reasonable to conclude that PEX generally triggers a stress response which leads to increased α B formation in the whole body. This exacerbates the imbalance between α A and α B expression in the eye lens to such an extent that it must be assumed that the hetero-oligomers necessary for the protection of the eye lens can no longer be formed (**Figure 4D**).^{36–40}

We conclude that the human lens is affected by PEX in particular due to two pathomechanisms. Using our FLIM measurements, we were able to show that the specific lifetime of the PEX material differs significantly from that of both the lens capsule and the mitochondria-rich lens epithelial cells, which argues against local production. Thus, we believe that PEX deposits represent an abnormal, highly protein-rich accumulation in the extracellular matrix that adheres to the lens capsule, as the aggregates are too large to drain away and consequently accumulate in the aqueous humour. This is in line with the clinical finding of PEX flakes on intraocular lenses. Our molecular biological investigations provide evidence that accelerated cataract development is triggered by changes in crystallin expression. This is, on the one hand, the result of increased α B expression, which shifts the physiological balance in relation to α A, and, on the other hand, the result of upregulation of the stress protein crystallin β B2.

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FIGURE LEGENDS

FIGURE 1. Slit lamp biomicroscopy photographs of **(A)** a 70 years old male phakic patient with PEX fibrils on the eye lens, **(B)** a 86 years old female pseudophakic patient on the artificial intraocular lens (IOL) and **(C, D)** a 86 years old female pseudophakic patient with dislocated IOL. The dislocated IOL is marked by asterisk and the loose zonule fibers with flake like chains of adherent PEX material by arrows. Magnifications are 10 x **(A-C)** and 25 x **(D)**.

FIGURE 2. Characterisation of lens AGEs content in three groups of patients: cataract (open circles and white bars), cataract and PEX (dark grey circles and bars), and cataract and diabetes (light grey circles and bars). **(A)** AGEs content per lens sample depending on age of the patient for each group was correlated. **(B)** Mean AGEs content (ng/ μ g protein), **(C)** mean best-corrected visual acuity (LogMAR), and **(D)** mean central retinal thickness (μ m) were compared between groups. Data are expressed as mean $\pm$ SEM of n = 11 (cataract), n = 10 (cataract and PEX), and n = 8 (cataract and diabetes) patients; **P < 0.01, ****P < 0.0001 (ANOVA/Bonferroni's test).

FIGURE 3. Crystallin gene expression was determined in three groups of patients: cataract (open circles and white bars), cataract and PEX (dark grey circles and bars), and cataract and diabetes (light grey circles and bars). Relative expression to actin of **(A)** CRYAA, **(B)** CRYAB, **(C)** CRYBB1, **(D)** CRYBB2 and **(E)** CRYGC was calculated from duplicate measurements per lens sample. Data are expressed as mean $\pm$ SEM from lens samples of n = 11 (cataract), n = 10 (cataract and PEX), and n = 8 (cataract and diabetes) patients; *P < 0.05, **P < 0.01 (ANOVA/Bonferroni's test).

FIGURE 4. Ratio of crystallin A gene expression in patients with cataract (open symbols and white bars) and cataract and PEX (dark grey symbols and bars). **(A)** Sum of relative gene expression to actin of CRYAA and CRYAB, **(B)** Ratio of CRYAA to CRYAB and **(C)** individual expression of CRYAA and CRYAB in relation to the expression of the sum of CRYAA and CRYAB. Data **(A, B)** are expressed as mean $\pm$ SEM; * $P < 0.05$ (Student's *t*-test). **(D)** Schematic diagram illustrating the unphysiological overload of CRYAB in PEX.

FIGURE 5. Characterisation of the inside and outside of the anterior lens capsule by TPEF. **(A)** Inside showing epithelial cells and **(D)** outside showing PEX fibrils of the lens capsule of a 65 years old patient with cataract and PEX. Inserts with enlarged image section give a deeper view. Inside of a lens capsule with epithelial cells from **(B)** a 77 old patient with cataract and **(C)** a 62 old patient with cataract and diabetes. **(E)** Sticked image shows the outside of the whole anterior lens capsule piece removed by cataract surgery of a 74 years old patient with cataract and PEX. Squares 1 and 2 marked by a dashed line are showed enlarged the PEX fibrils. Fluorescence signal obtained by excitation with 740 nm is shown in gray. Scale bars and 3D blend projection as indicated.

FIGURE 6. TPEF images of the anterior lens capsule of a 76 years old patient with cataract and PEX. **(A)** In addition to PEX fibrils adhering to the lens capsule, also floating material is shown. PEX fibrils of the enlarged area are shown in **(B)** as resolved 3D and **(C)** as separated bottom, center and top layer to provides a detailed view of the structure. Fluorescence signal obtained by excitation with 740 nm is shown in gray. Scale bars as indicated.

FIGURE 7. FLIM imaging of the inner side of the anterior lens capsule showing epithelial cells in a 73 years old patient with cataract. **(A)** Lens epithelial cells were detectable by their characteristic metabolic autofluorescence. Lifetime color map shows shorter lifetime component in blue and longer lifetime component in red. **(C)** Pattern 1, blue; mitochondria-rich cellular cytoplasm $\tau_1 = 3.334$ ns, vs **(B)** pattern 2, red; capsule basement membrane $\tau_2 = 3.917$ ns **(D)** Phasor plot with pattern separation. Dark nuclear centers confirm epithelial identity. Scale bar: 5 μ m **(E)** Three representative fields of view showing autofluorescence from lens epithelial cells in a sample of the control group overlying the anterior capsule. **(F)** Three corresponding fields of view acquired and displayed with identical settings in a sample of the control group, demonstrating a similar epithelial cell and capsule lifetime pattern. Together, panels E and F define the baseline lifetime signature of the anterior lens epithelium-capsule complex in eye lens of the control group. Scale bars as indicated.

FIGURE 8. FLIM imaging of the outer side of the anterior lens capsule showing PEX fibrils in a 71 years old patient with cataract and PEX. **(A)** PEX fibrils showed a different lifetime compared to the capsule basement membrane; lifetime color map shows shorter lifetime component in blue and longer lifetime component in red. **(C)** Pattern 1, blue; PEX material, $\tau_1 = 3.838$ ns, vs **(B)** pattern 2, red; capsule

basement membrane $\tau_2 = 4.232$ ns **(D)** Phasor plot with pattern separation. Scale bar: 5 μ m. **(E-F)** Granular PEX material morphology in two other representative patients of the PEX group. Scale bars as indicated.

FIGURE 9. Comparison of fluorescence lifetimes of anterior lens capsule, mitochondria and cytoplasm of epithelial cells and PEX deposits. Fluorescence lifetime for each component was calculated from 7 independent images exemplarily shown in Figures 7 and 8. The lifetime of PEX deposits is significantly different from mitochondria of epithelial cell and the lens capsule. Data are expressed as mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001 ****P < 0.0001 (ANOVA/Bonferroni's test).

FIGURE 10. FLIM images of the cut edge of the anterior lens capsule of a 65 years old patient with cataract and PEX. **(A)** FLIM image of the capsule margin (left) with granular PEX deposits overlying the capsule basement membrane and corresponding three-pattern phasor segmentation (right): Pattern 1 (blue), PEX material, $\tau_1 = 4.011$ ns; pattern 2 (red), cell (cytoplasm), $\tau_2 = 4.704$ ns; pattern 3 (green), lens capsule, $\tau_3 = 4.936$ ns **(B)** Second FLIM field from the same specimen, again showing granular PEX deposits on the capsule (left) and corresponding pattern-matching map (right): Pattern 1 (blue), PEX material, $\tau_1 = 4.049$ ns; pattern 2 (red), cell (cytoplasm), $\tau_2 = 4.461$ ns; pattern 3 (green), lens capsule, $\tau_3 = 4.685$ ns. **(C)** Peripheral zoom of the right side of panel B, highlighting epithelial cells at the capsule surface: Pattern 1 (blue), epithelial cells, $\tau_1 = 4.207$ ns; pattern 2 (red), capsule basement membrane, $\tau_2 = 4.839$ ns. **(D)** Corresponding table of lifetime values of **(A)**, **(B)** and **(C)**. Scale bars as indicated.

FIGURE 11. Schematic diagram illustrating the characterization of PEX material using high-resolution, label-free microscopy techniques FLIM and TPEF.

Figure 1

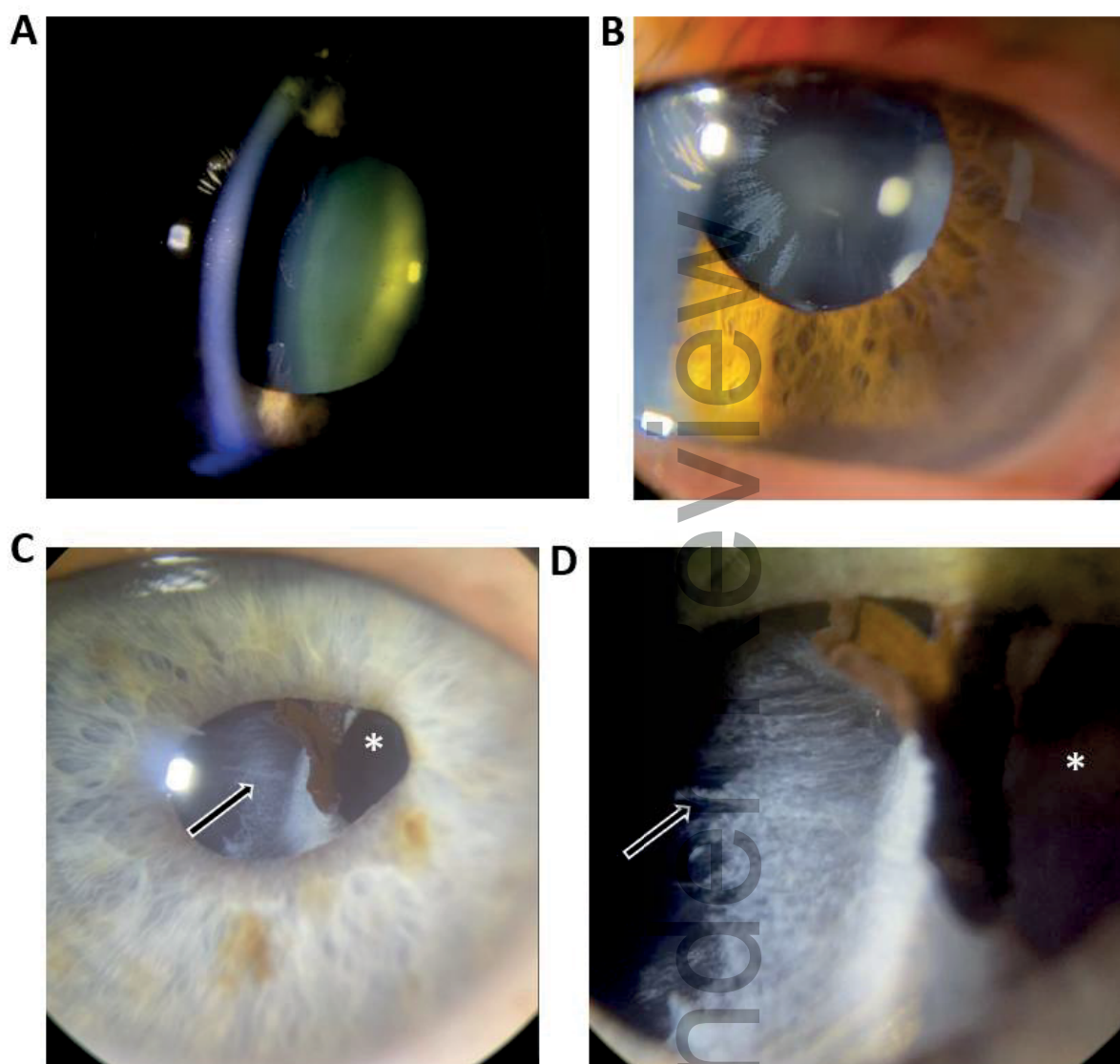


Figure 2

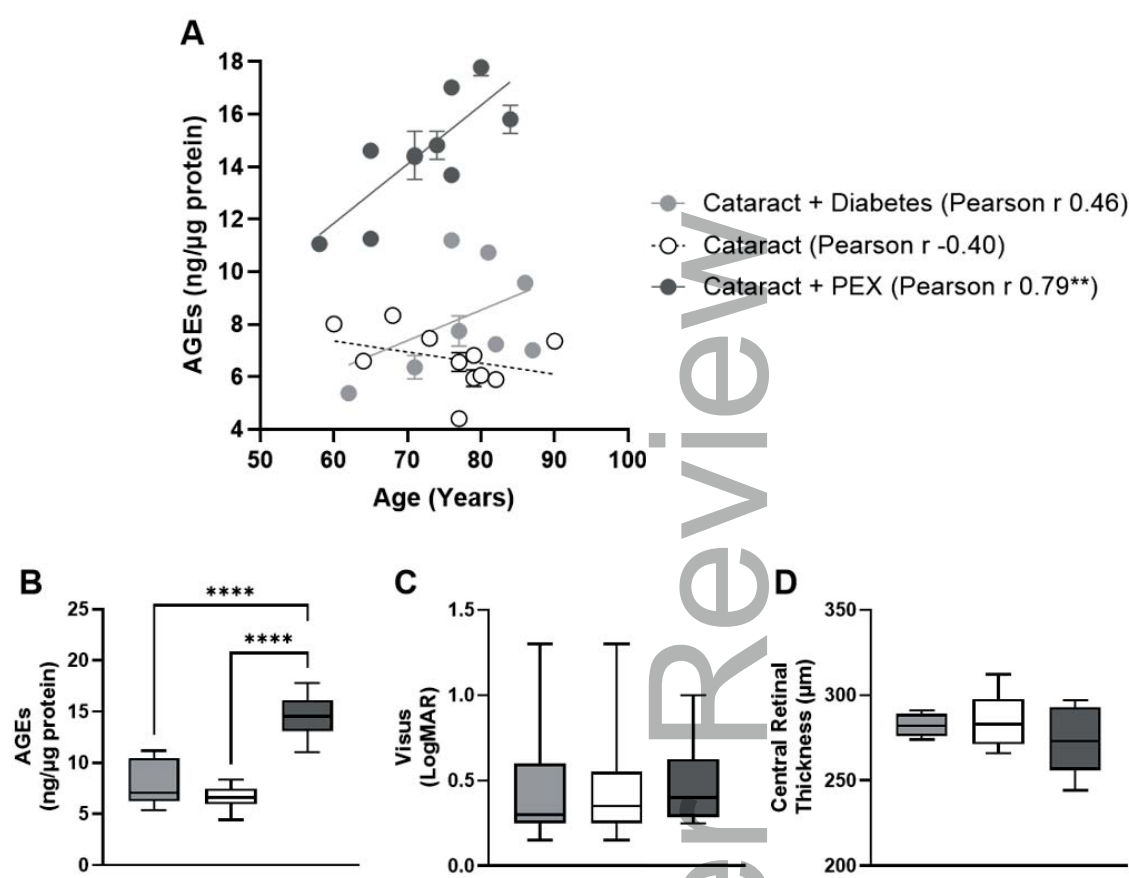


Figure 3

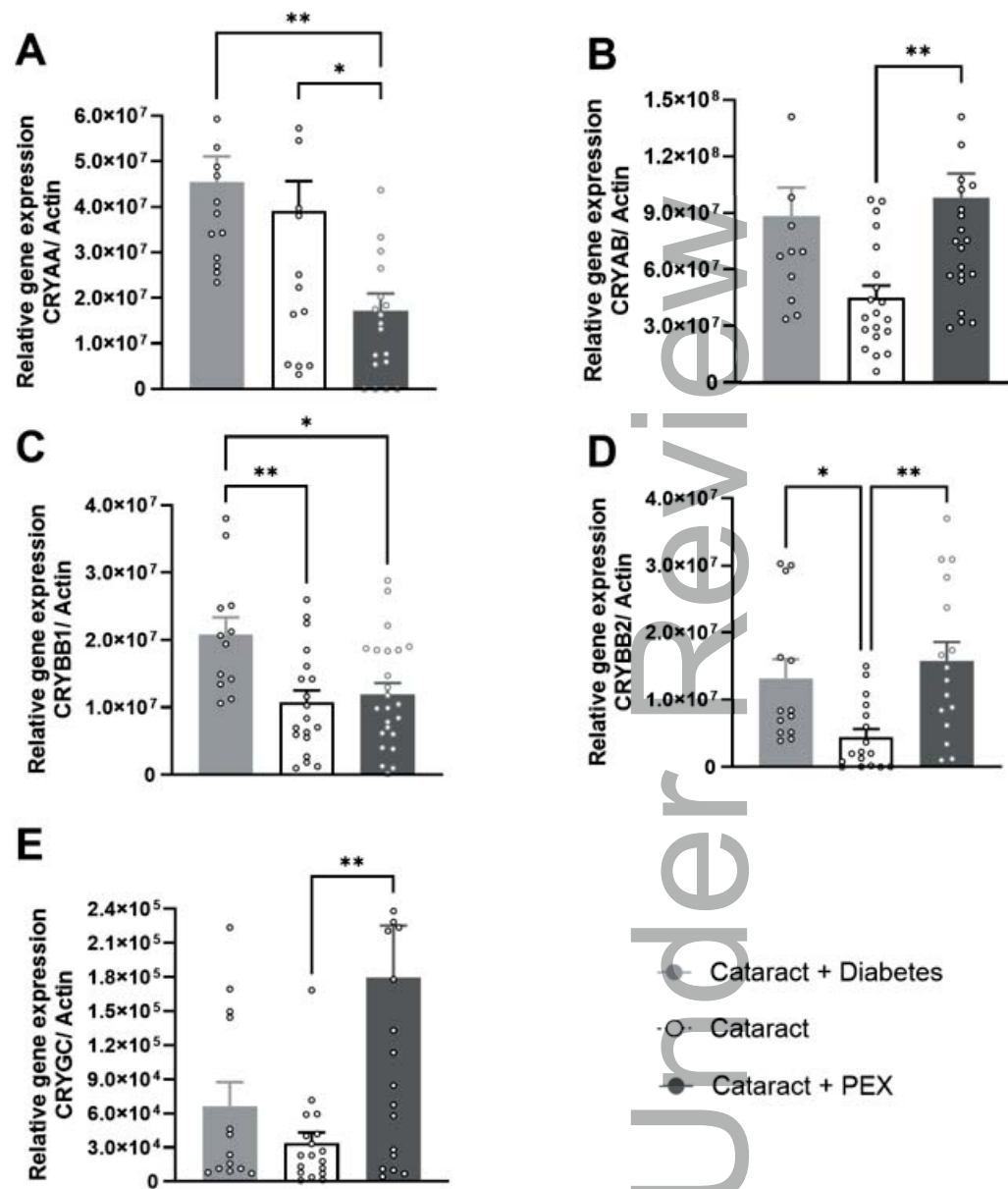


Figure 4

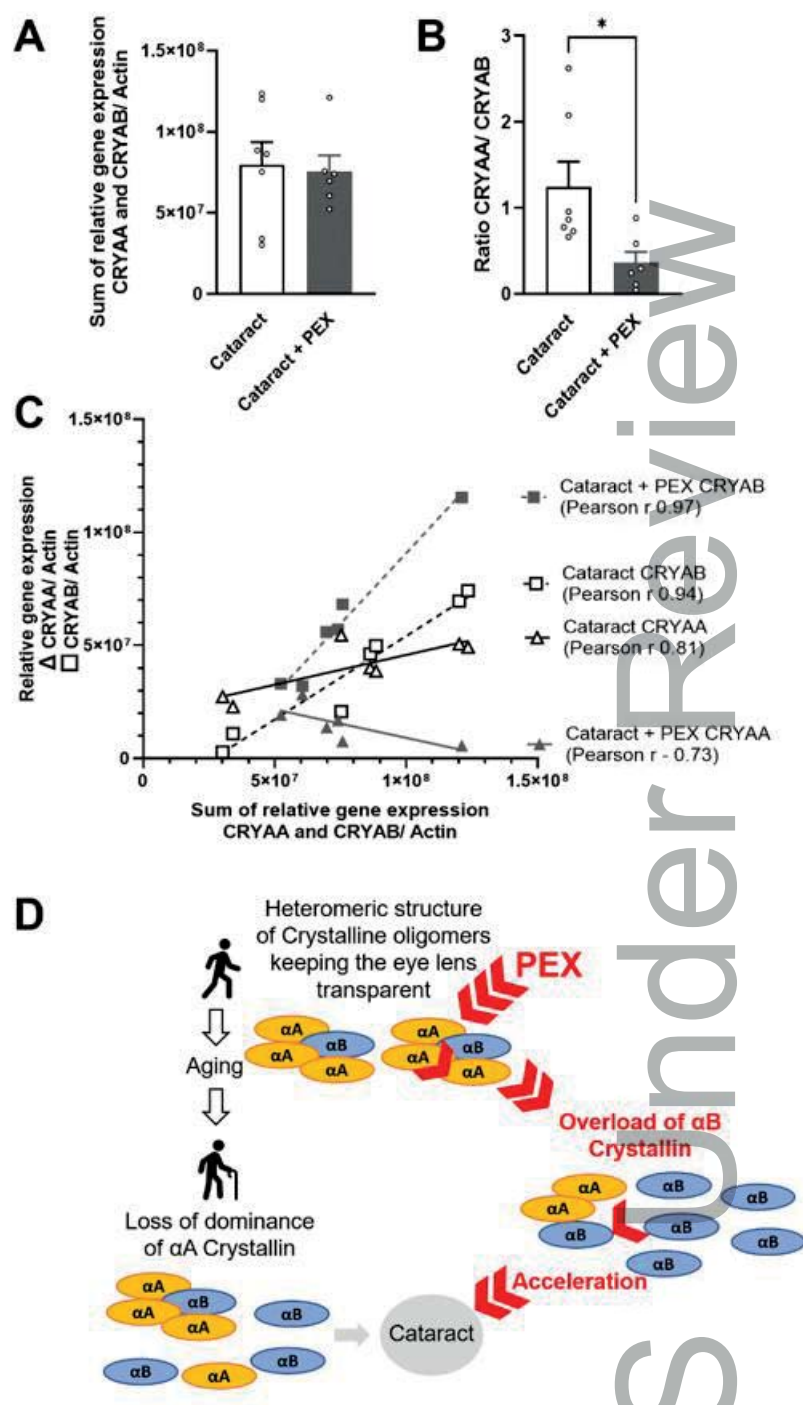


Figure 5

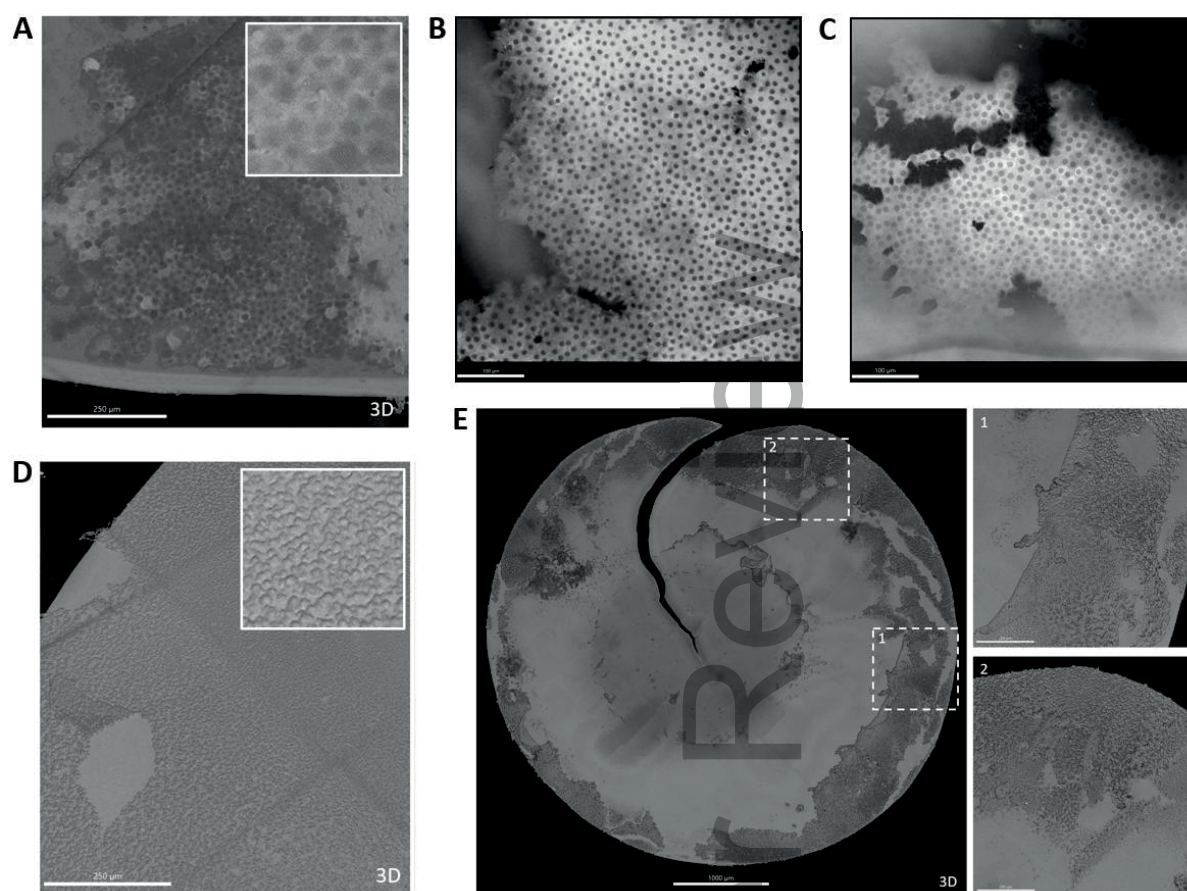


Figure 6

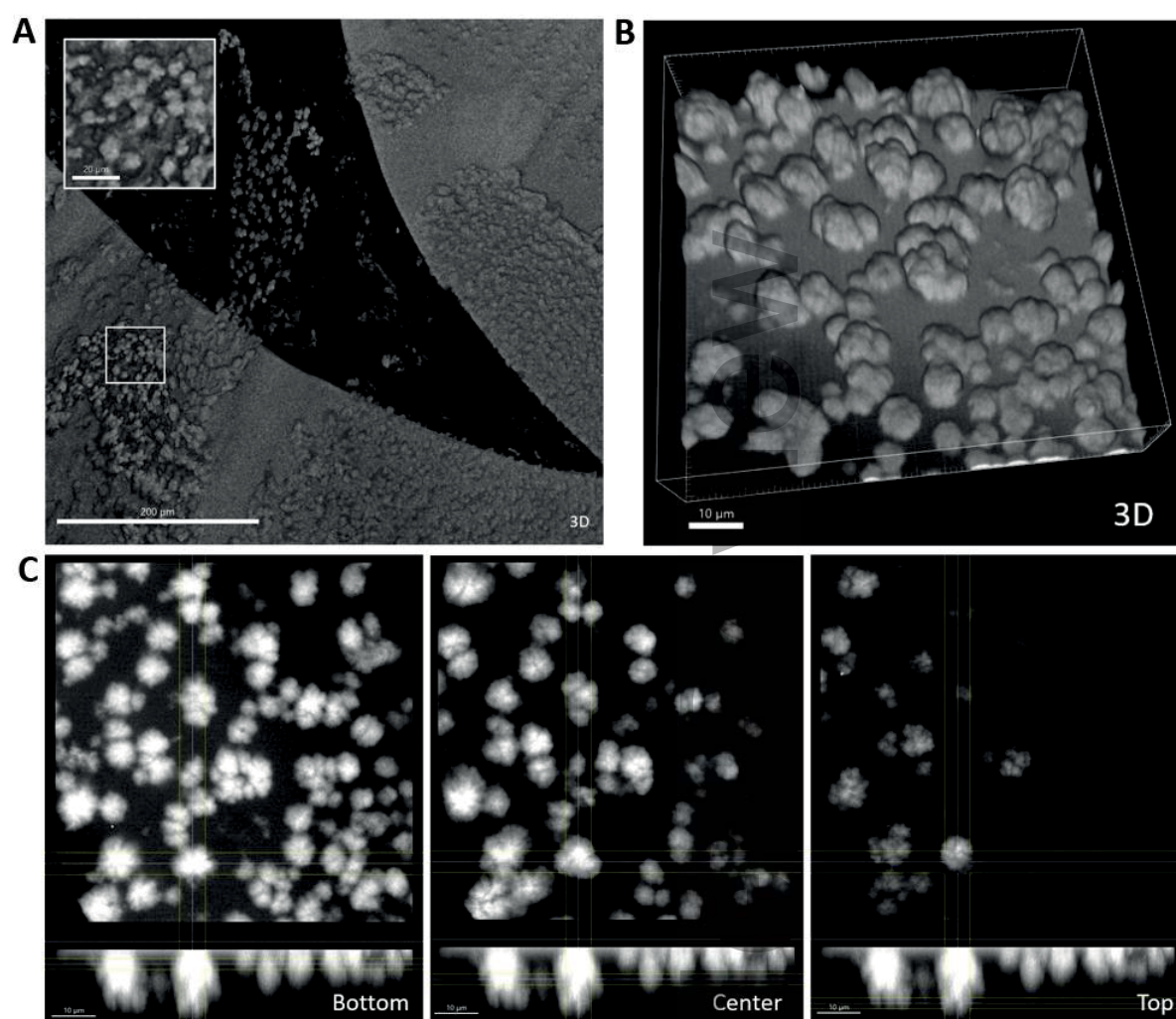


Figure 7

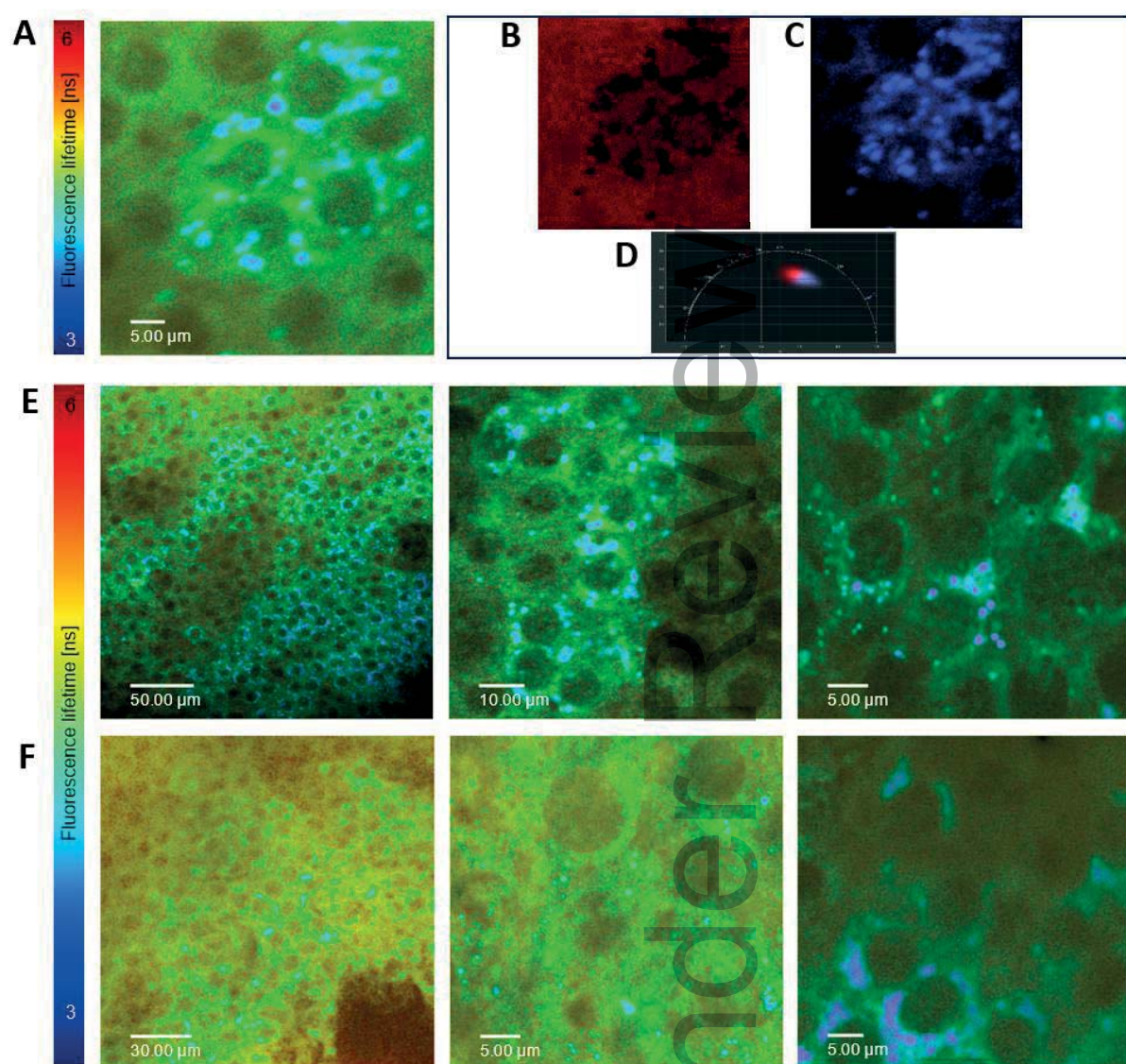


Figure 8

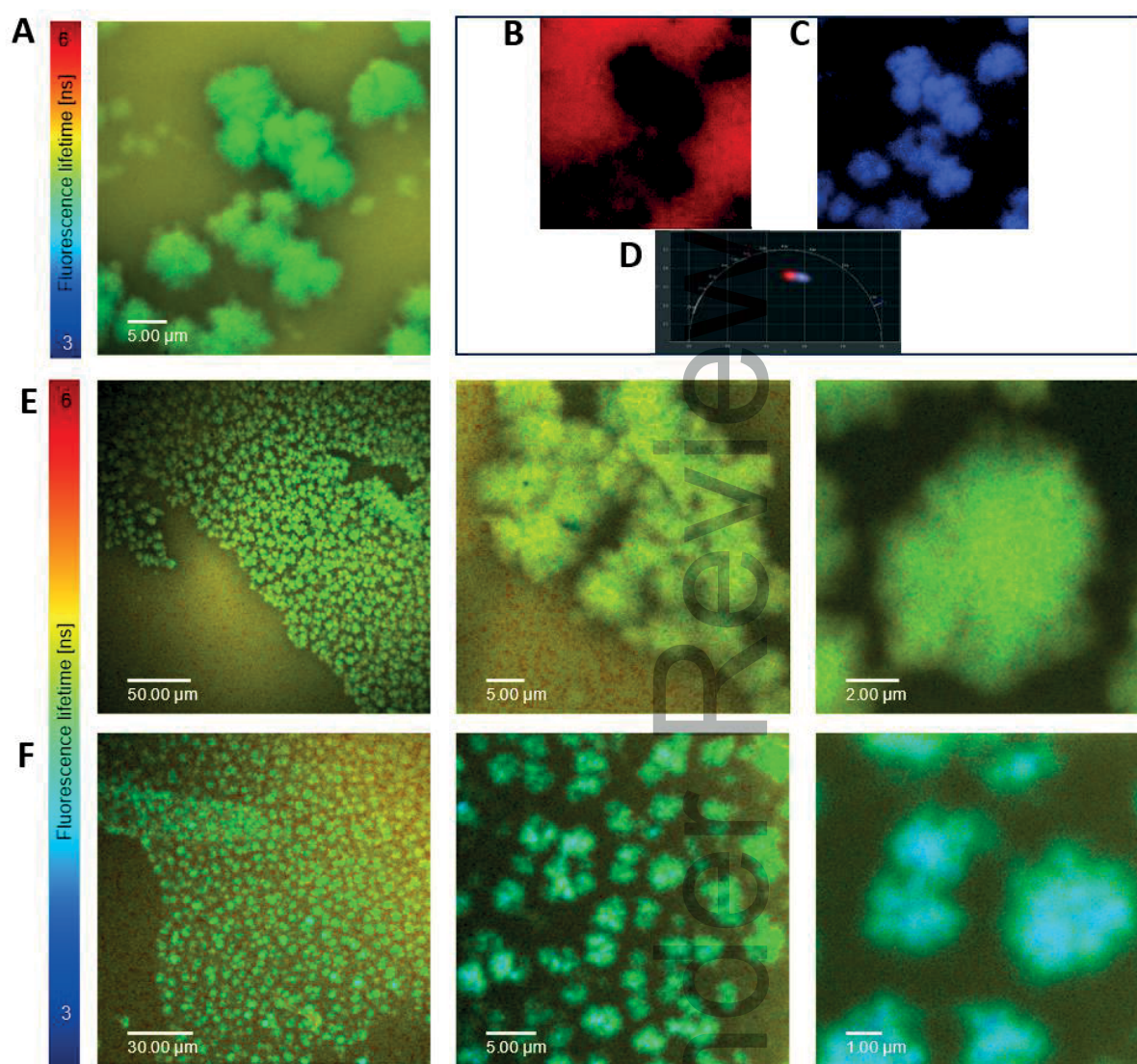


Figure 9

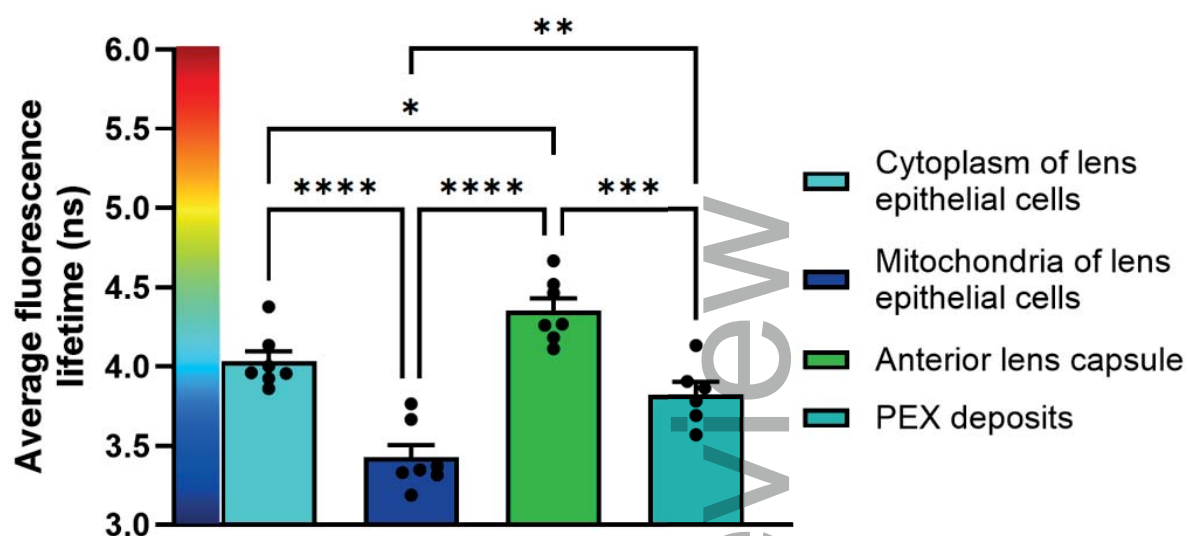


Figure 10

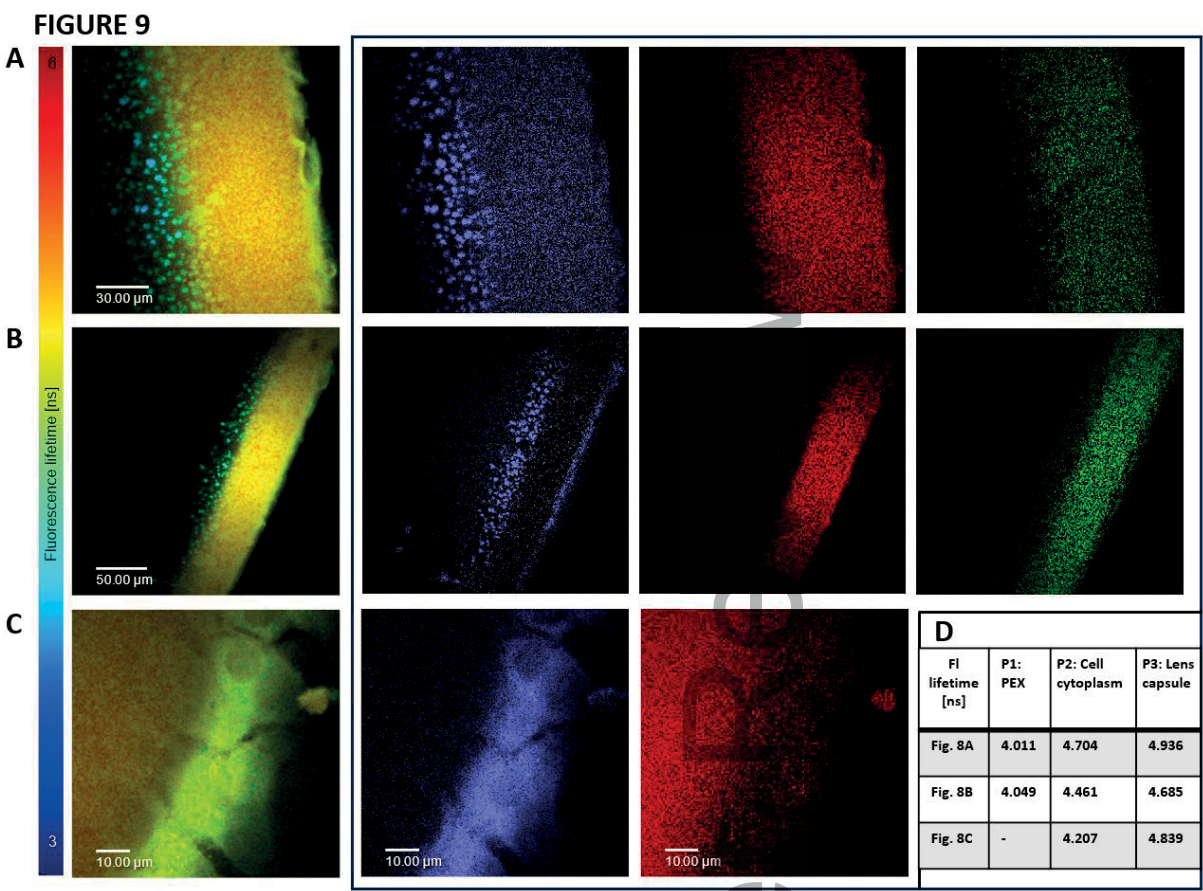


Figure 11

